CDRI-WRAIR Collaborative Project

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COMPARATIVE DRUG RESPONSES OF SENSITIVE AND RESISTANT STRAINS OF MALARIAL PARASITES USING IN VITRO BIOASSAYS AND ANIMAL MODELS FOR BLOOD AND TISSUE SCHIZONTOCIDAL ACTIVITY AND MECHANISM OF REVERSAL OF RESISTANCE AND TOXICITY OF ANTIMALARIALS

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A shorter 3 day treatment regimen of compound WR 238605 for anti-relapse activity has been established using P.cynomolgi B model. This compound is undergoing Phase I clinical trials at Walter Reed. This compound has also been found to show significant blood schizontocidal activity as shown by curative action on blood induced P.cynomolgi B and P.fragile infection. A new anti-relapse therapy for possible use in chloro- quine resistant P.vivax areas has been successfully developed in which anti-relapse compound WR 238605 is given with halofantrine as blood schizontocide instead of chloroquine. This will be of potential value for use in chloroquine resistant P.vivax areas. The results also suggest that the two drugs would have synergistic effect as blood schizontocide. An isolate of mefloquine, resistant P.knowlesi has been developed which would be used for mefloquine resistance reversal studies. Three drugs evaluated for resis- tance reversal potential against multiple drug resistant rodent model did not show any promising results. Tissue stages of P.cynomolgi have been obtained after successful inoculation of hepatocyte cultures with sporozoites.The cultivation of primary e-e stages will be useful to develop in vitro model for screening of tissue schizontocidal drugs.

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23 Feb, 1995

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SUMMARY

1. CYCLIC TRANSMISSION OF P. CYNOMOLGI B:

Plasmodium cynomolgi B is being maintained by cyclic transmission through A.stephensi and sixteen serial passages (Sr. Nos. 87-102) were conducted during the period of report.

2. REVALIDATION OF CHLOROQUINE AND PRIMAQUINE CURATIVE DOSES:

The curative blood schizontocidal dose of chloroquine and causal prophylactic and anti-relapse curative doses of primaquine have been revalidated and no escalation in curative dose established earlier has been observed. The protocols for blood schizontocidal, causal prophylactic, anti-relapse, gametocytocidal/sporontocidal efficacy tests are operational.

3. BLOOD SCHIZONTOCIDAL CURATIVE DOSE OF CHLOROQUINE IN THE SHORTER THREE DOSE REGIMEN:

Curative dose of chloroquine has also been determined using shorter three dose treatment schedule and the dose of 10.0 mg/kg x 3 days has been found to be curative.

4. ADDITIONAL DATA ON BLOOD SCHIZONTOCIDAL ACTIVITY OF WR 238605:

Additional blood schizontocidal data has been obtained for compound WR 238605 against two simian malaria parasites namely P. cynomolgi B and P. fragile and the new compound has shown 10 fold better blood schizontocidal activity than primaquine.

5. GAMETOCYTOCIDAL ACTIVITY OF WR 238605:

Compound WR 238605 has also shown significant gametocytocidal activity at 2mg/kg single dose against P. cynomolgi B.

The compound has been selected by Walter Reed for Phase I clinical trials. This c ompound is a potential anti-relapse antimalrial which may eventually replace primaquine.

6. RADICAL CURATIVE ACTIVITY OF WR 238605 IN THE SHORTER THREE DOSE REGIMEN:

Compound WR 238605 was evaluated for anti-relapse activity using three dose treatment regimen and a dose of 1.00 mg/kg x 3 days was found curative.

7. BLOOD SCHIZONTOCIDAL ACTIVITY OF 3 ANTIMALARIALS:

The curative blood schizontocidal dose of mefloquine (10mg/kgx7), halofantrine (10mg/kgx7) and WR 242511 (1.00 mg/kgx7) has been established against P. cynomolgi B in rhesus monkey.

8. BLOOD SCHIZONTOCIDAL ACTIVITY OF HALOFANTRINE AND WR 238605 COMBINATION:

Combination of 0.316 mg/kg WR 238605 and 3.16mg/kg halofantrine has been found to be curative against blood induced P. cynomolgi infection. The results suggest that the two drugs are having additive/synergistic effect.

9. RADICAL CURATIVE ACTIVITY OF WR 238605 USING HALOFANTRINE AS THE COMPANION BLOOD SCHIZONTOCIDE

The radical curative dose of compound WR 238605 has been evaluated in combination with halofantrine as the companion blood schizontocide. The dose of 0.316 mg/kg has been found curative and there has been no change in the dose when halofantrine instead of chloroquine was used as the companion drug.

10. in vitro CULTIVATION OF P. FALCIPARUM:

Technology for in vitro cultivation of P. falciparum to establish in vitro antimalarial screening protocols is being standardized using Giemsa staining of culture smears to monitor the parasiticidal dose end point. Standardization of in vitro bioassay for anti-malarials using tritium labelled Hypoxanthine incorporation will be planned in the next year.

11. IN VITRO TISSUE SCHIZONTOCIDAL SCREENING MODEL

For in vitro screening of prospective tissue schizontocides, technology to obtain primary hepatocyte cultures and development of exoerythrocytic stages after inoculation of sporozoites has been established. Primaquine at 0.1ug/ml has been found to inhibit development of e-e schizonts inhibit development of e-e schizonts.

12. DRUG RESISTANT STRAINS FOR RESISTANCE REVERSAL STUDIES

(a) Simian malaria:

The following sub lines of P. knowlesi W_1 have been initiated with a view to establish stable drug resistance.

(1) Chloroquine resistant strain: Efforts are continuing to establish chloroquine resistant strain of P. knowlesi.

(2) Mefloquine resistant strain: has been developed and it can tolerate upto 80mg/kgx3day dose and will be useful for evaluation of mefloquine resistance reversal agents such as penfluoridol and other potential reversal agents. The mefloquine resistant P. knowlesi has been cryopreserved.

(b) Rodent malaria:

(i) The following drug resistant lines of rodent malaria parasite P. berghei have been cryopreserved.

1. Chloroquine resistant strain (upto 128mg/kgx4)
2. Mefloquine resistant strain (upto 128mg/kgx4)
3. Quinine resistant strain (upto 400mg/kgx4)

(ii) A multiple resistant strain of P. voelii nigeriensis resistant to chlolroquine (128mg/kgx4), mefloquine (128mg/kgx4) and quinine (400mg/kgx4) has been cryopreserved. This strain has been used for resistance reversal studies. This strain produces 100% lethal infection.

13. STUDIES ON REVERSAL OF DRUG RESISTANCE:

Several resistance reversal agents have been published in literature but in most of the studies the reversal effect was observed against cultures of chloroquine resistant P.falciparum. Studies have been initiated to validate the resistance reversal effect in multiple drug-resistant rodent malaria model (P.yoelii nigeriensis).

(i) WR 238605 + Chloroquine combination:

The marginal extension of MST by 2 days was observed when WR 238605 (0.5 mg/kg) was given together with chloroquine (4.0 or 8.0 mg/kg) as compared to MST of control chloroquine group suggesting some additive effect of the WR 238605 when combined with chloroquine.

(ii) WR 238605 + Mefloquine combination:

WR 238605 (0.5mg/kg) did not potentiate the effect of mefloquine against mefloquine resistant

strain.

Verapamil:

Verapamil at higher doses provided a definitive extension of MST when given the drug was given together with chloroquine. Studies shows a limited chloroquine resistance reversal effect of Verapamil in day 3-6 treatment schedule.

Nifedipine:

Nifedipine at 10-15mg/kg given with chloroquine 8mg/kg resutled in extension of mean survival time to 24.7-24.8 days compared to 21.14 days of chloroquine control group, indicating some chloroquine resistance reversal effect of Nifedipine against multiple resistant rodent model used in this study.

PROGRESS OF WORK

1. CYCLIC PASSAGE OF P. CYNOMOLGI B:

Since the initiation of collaborative WRAIR-CDRI project in 1982 the simian parasite P. cynomolgi B has undergone 102 cyclic passages. ?The details of last sixteen serial passages (87-102) are summarized (Table 1 and 2) and the parasite has given high infectivity in Anophelese stephensi (colony bred). The insectary is maintaining 2000-3000 pupae/day under standard insectary conditions. Adequate numbers of sporozoites can be produced for accomplishing the tasks involved in prophylactic and radical curative tests. Invariably the patency after sporozoite inoculation ($0.73x10^6$ to $1.54x10^6$) has been recorded in thick smears on day 8-9.

2. REVALIDATION OF CURATIVE DOSE OF STANDARD DRUGS:

a. Chloroquine:

Revalidation of chloroquine curative blood schizontocidal dose has been carried out at a dose of 3mg chloroquine base/kgx7 days in 2 monkeys using parasite inoculum from 94th serial passage. This dose has been found to be curative in both monkeys (Table-3).

Three day regimen: Three day regimen of chloroquine was evaluated against blood induced infection of P. cynomolgi B using 5.0; 7.5 and 10.0 mg/kg doses of chloroquine (base) administered orally for three consecutive days. Results in Table-4, show that 10.0 mg/kg was the curative dose, 7.5mg/kg was curative in one out of two monkeys and 5.0mg/kg failed in both the monkeys. The monkeys which showed recrudescence in the above study were again treated at 7.5mg/kg and 10.0mg/kg and both these doses were curative The curative dose will be revalidated because this shorter regimen is operationally more convenient.

b. Primaquine:

Prophylactive dose of primaquine (1.78mg base/kgx3 days) was revalidated against sporozoite induced P. cynomolgi B infection in 2 monkeys and both the monkeys were cured (Table-5).

Radical curative dose of primaquine (1mg/kg base x7days) was revalidated in 2 monkeys each during 86th and 90th serial passages and dose was found to be curative. The lower dose 0.316mg/kgx7 days used during 86th serial passage was not curative as expected and monkeys relapsed on day 29 and 37 (Table-6 and 7).

CONCLUSION:

The curative blood schizontocidal dose of chloroquine, causal prophylactic and radical curative doses of primaquine, have shown no escalation during the last 13 years.

3. ADDITIONAL ANTIMALARIAL DATA WITH COMPOUND WR 238605:

a. Blood Schizontocidal Activity:

The compound WR 238605 had earlier been evaluated for antirelapse (0.316mg/kgx7days) activity and causal prophylactic (0.316mg/kgx9days) activity. Additional data have been generated for its blood schizontocidal activity against two simian parasites viz. P. cynomolgi B and P. fragile. Results in Table-8 show that against P. cynomolgi B infection, 10 out of 12 monkeys were protected at 1mg/kg dose x7 days. All the 6 monkeys treated at 3.16mg/kgx7 were also cured. In comparison primaquine was curative in any of the 4 monkeys at 3.16mg/kgx7 and in 3 out of 4 monkeys at 10mg/kgx7 days. Likewise, against P. fragile infection, compound WR 238605 cured 10 out of 11 monkeys at 1mg/kg and all the 4 monkeys at 3.16mg/kgx7 days. Primaquine protected 1 out of 3 monkeys at 3.16mg/kgx7 and 2 out of 3 monkeys at 10mg/kg dose x7 days.

CONCLUSION:

Compared to the primaquine, compound WR 238605 has shown 10 fold higher blood schizontocidal activity against P. cynomolgi B and P. fragile infections in rhesus monkey models.

b. Gametocytocidal Activity of WR 238605 against P. cynomolg B:

For the gametocytocidal test, batches of 3-4 day old An. stephensi were allowed to feed on P. cynomolgi infected rhesus monkeys at appropriate gametocytaemia level. Our earlier studies have shown that the sequential feeding of healthy mosquitoes for consecutive 3-4 days during the declining phase of the secondary asexual peak parasitaemia gave consistently good infectivity. One hr after the control (pre-treatment) feeding, compound WR 238605 was administered to the monkey at 1.0, 2.0 and 4.0 mg (base)/kg in a single dose by oral route. Post-treatment feeding batches of healthy mosquitoes was done at different times (6-48 hr). Mosquitoes were maintained at 27+1°C under optimal insectary conditions. The infectivity rate and the oocyst counts were recorded on day 8. Mosquitoes were further, maintained in the insectary upto day 15 to determine the formation of sporozoites in the experimental batches.

RESULTS

The gametocytocidal activity of WR 238605 was evaluated in 7 rhesus monkeys and the pre-treatment mosquito infectivity results for these monkeys show that the oocyst number of different batches ranged from 17.13 + 10.01 to 35.32 + 13.34 and the percent infectivityu varied between 64.10 to 86.49% (Table-9). Sequential mosquito feedings on three monkeys treated at 1.00;mg/kg dose showed that there was no significant reduction in oocyst number and the percent infectivity in +6 hr mosquito batches for all the three monkeys and in +24 hr post treatment batches for two out of three monkeys when compared to the corresponding control feeding at -1 hr. Salivary gland dissections of the mosquitoes from these batches on day 15 showed the presence of sporozoites, thus indicating that

oocysts completed normal sporogonic development. No oocysts were observed over the midguts from mosquitoes fed at +48 hr after drug administration nor were any sporozoites seen in their salivary glands.

Identical results were obtained in the efficacy tests at 2.0mg/kg in 3/3 monkeys and at 4.0mg/kg in one monkey. The mosquito batches fed at +6 hr post-treatment showed no significant alteration in the oocyst numbers, and these oocysts were able to complete the sporogonic cycle as indicated by the presence of sporozoites in salivary glands on day 15-16. The mosquito batches fed on these monkeys at +24 hr did not develop any oocysts nor were any sporozoites demonstrable in their salivary glands.

c. Shorter Three Dose Regimen for Radical Curative Activity:

Compound WR 238605 had been earlier found to show anti-relapse activity at 0.316mg/kg dose in the seven day regime :. Studies were carried out to determine the curative dose of the compound in "**Three dose regimen**". Two monkeys each were treated at 0.50mg/kg, 1.0mg/kg and 2.0mg/kgx3 days. Results show that both monkeys at 0.50mg/kg relapsed on days 25 and 43, while monkeys treated at higher doses were protected (Table10). Further revalidation studies of the three dose regimen will be carried out.

4. BLOOD SCHIZONTOCIDAL ACTIVITY OF MEFLOQUINE, HALOFANTRINE AND WR 242511:

a. Blood Schizontocidal Activity of Mefloquine:

The blood schizontocidal activity of mefloquine was evaluated in 2 monkeys each at 3.16mg/kg., 10mg/kg and 31.6mg/kgx7days. The lower dose of 3.16mg/kg failed to clear the parasitaemia in both the monkeys while monkeys treated at higher doses were cured and no recrudescence was observed till 60 days (Table-11). The dose of 10mg/kg was revalidated in 2 naive monkeys and both were protected (Table-12).

b. Blood Schizontocidal Activity of Halofantrine:

The blood schizontocidal activity of Halofantrine was evaluated in 2 monkeys each at 3.16mg/kg, 10.00 and 31.6mg/kgx7 days. The lowest dose of 3.16mg/kg was not curative as indicated in Table-13. Monkeys at the higher dose i.e. 10.00 and 31.6mg/kgx7 days were cured and did not show any recrudescnce. Activity at 10mg/kgx7 days was revalidated in 2 monkeys (Table-14), and it was found to be curative. further tests were carried out at 5.6mg/kgx7 days dose schedule in four monkeys, and the compound was curative at this dose in three out of four monkeys, while the fourth showed recrudescence. Test carried out at 10mg/kgx7 days, was curative in both the monkey (Table-15).

c. Blood Schizontocidal Activity of WR 242511:

The blood schizontocidal activity of WR 242511 was evaluated in 2 monkeys each at 0.316 mg/kg, 1.00 mg/kg and 3.16mg/kgx7 days. The lowest dose of 0.316mg/kg was not curative as indicated in Table-16. Monkeys at the doses of 1.00 and 3.16mg/kgx7 days were cured and have not shown recrudescence during 60 days observation period. Revalidation of 1mg/kgx7days dose showed that the dose was curative in two monkeys (Table-17).

5. COMBINATION STUDIES WITH COMPOUND WR 238605 AND HALOFANTRINE:

In view of the sporadic emergence of chloroquine resistant P. vivax parasites, the treatment of resistant cases would need the shifting of chloroquine therapy to an alternate blood schizontocide for use as companion drug with the radical curative agent like primaquine or the new compound WR 238605 which is under clinical phase I trials at Walter Reed. Amongst the alternate blood schizontocides which can replace chloroquine include mefloquine and halofantrine. With a view to establish their efficacy, the data generated with these compounds clearly show that mefloquine and halofantrine are individually curative as blood schizontocides at 10mg/kgx7 days schedule. In contrast

to these, chloroquine curative dose has been again established against P. cynomolgi B and it has been found to be 3.00mg/kgx7 days dose.

Further studies have been carried out using halofantrine in combination with the anti-relapse antimalarial WR 238605 in both the blood schizontocidal test and the radical curative test with a view to see whether the combination has additive/synergistic/antagonistic effect.

a. Blood Schizontocidal Activity of WR 238605 + Halofantrine Combination:

Studies with these compounds when used individually have shown that compound WR 238605 is curative at 3.16mg/kgx7 days and halofantrine is curative at 10.0mg/kgx7 days. Concurrent administration of WR 238605 at 0.316m/gkg and halofantrine at 3.16mg/kgx9 protected two out of two monkeys, while WR 238605 at 0.316mg/kg in combination with halofantrine at 1.00mg/kg was not curative in any of the two monkeys (Table-18). Results indicate that the combination shows additive/synergistic effect as the curative doses of the components in the combination have been lowered by 10 and 3 fold respectively.

b. Radical Curative Activity of WR 238605 and Halofantrine:

To evaluate the anti-relapse efficacy of Wr 238605 in combination with halofantrine as the companion blood schizontocide, two monkeys each were treated with a combination of WR 238605 and halofantrine at 0.316mg/kg + 3.16mg/kg, 0.316mg/kg + 5.62mg/kg and 0.316mg/kg + 10.0mg/kgx7 days respectively. Follow up of these monkeys till 100 days showed that none of the monkeys relapsed thus indicating the curative efficacy of the doses (Table-19). In the second experiment, compound WR 238605 at 0.316mg/kg dose was evaluated in combination with halofantrine at 1.78mg/kg, 3.16mg/kg and 5.62mg/kg doses, in two monkeys each. While one of the monkey at the lowest dose(0.316 + 1.78mg/kg) relapsed on day 26, other monkeys have not shown any relapse till day 30 (Table-20). One monkey treated with compound WR 238605 alone at 0.316mg/kg relapsed on day 26. Additional two monkeys treated with WR 238605 at 0.1mg/kg and halofantrine at 10mg/kg also relapsed on day 13 and 15. Follow up of the other monkeys upto day 100 will continue.

6. in vitro CULTIVATION OF AND ANTIMALARIAL SCREENINGS:

a. Establishment of Technology for in vitro Cultivation of P.falciparum:

Technology for long term in vitro cultivation of Plasmodium falciparum (NF-54) strain has been established. The parasite was cultured in medium RPMI-1640 supplemented with 2% glucose and 10% 0+ human serum. Subcultures were done with human 0+ erythrocytes. The parasite was successfully cultured for 25 continuous cycles.

b. In vitro Micromethod for Testing of Antimalarials:

Micro-culture technique in 96 well plate was standardized to study the antimalarial action of new compounds. Assay was standardized using standard drug chloroquine. Serial dilutions of the drug were added in duplicate wells of 96 well plate. Cultured parasite was added at the ring stage of development. Effect of drug was monitored by making smears at 24 hrs and 48 hrs. Chloroquine completely inhibited parasite development at concentrations above 20ng/ml. Further studies are in progress to determine the lethal dose of other standard antimalrials on in vitro cultured parasites.

c. In vitro Testing for Tissue Schizontocidal Action:

A method has been standardized for primary screening of prospective tissue schizontocides using P. cynomolgi exoerythrocytic stages cultured in rhesus hepatocytes. Assay was standardized using standard tissue schizontocidal drug primaquine. The drug was added 24 hrs after sporozoite invasion of cultures. Primaquine exerted tissue schizontocidal action against the primary EE stages of the parasite at concentrations as low as 0.1ug/ml. Simultaneous experiments showed that chloroquine didn't exert any parasiticidal effect even at concentrations of 5ug/ml.

This assay will be useful for primary screening of tissue schcizontocides and will go a long way to replace the costly in vivo rhesus monkey model for conducting large scale evaluation of potential tissue schizontocides. This study will also provide new leads for identification of the site of action of

tissue schizontocides.

7. SELECTION OF CHLOROQUINE RESISTANT STRAIN OF P. KNOWLELSI:

a. Selection by relapse technique:

Attempts were made to select a chloroquine resistant strain of P. knowlesi W_1 by sequentially treating the infected monkeys at high parasitaemia level and the surviving parasites were inoculated into naive monkeys 24-48 hr after drug exposure. In the first passage, a monkey was treated at 25 mg total dose. The drug dose was gradually increased in 12 successive passages over a period a 174 days and a dose of 150 mg (total dose) was administered in the 12th passage. Several isolates were cryopreserved in different passages to check the chloroquine sensitivity at intervals. The parent strain (W_1) of P. knowlesi has been found to be curative at 7.5mg base/kg chloroquine x3 days. The chloroquine sensitivity of isolates cryopreserved during 11th passage was determined at 10.0, 15.0 and 20.0 mg/kgx3 days. The results showed that the parasite was resistant to a dose of 10mg/kgx3 as treated monkey recrudesced 11 days after end of treatment. The level of resistance was revalidated in two monkeys and stable resistant line could not be established.

b. Selection by interrupted subcurative therapy:

Attempts have also been made to select a chloroquine resistant strain of P.knowlesi by administering subcurative doses of chloroquine at interrupted intervals so as to allow constant drug exposure to the parasites. The first rhesus (Rh-1) was exposed to 5 doses of chloroquine ranging between 0.5-0 3 mg/kg during 8 days after which parasites were transferred to the naive monkey (Rh-II). Rhesus Rh-II was exposed to 25 doses of chloroquine ranging between 0.2-0.3 mg/kg. The parasite has been subsequently passaged in four naive monkeys Rh III, Rh IV, Rh V and Rh VI as indicated in Fig. I-VI and the subcurative chloroquine therapy was continued (Table-21). The strain has been under constant drug pressure since January 1994. The periodic sensitivity tests performed periodically 3 times indicated no escalation of chloroquine curative dose of 7.5mg/kg chloroquine basex3 days. The parasite has been under drug pressure for more than a year in rhesus monkeys.

8. SELECTION OF MEFLOQUINE RESISTANT P. KNOWLESI IN RHESUS:

Monkey:

Three rhesus monkey No. 1,2 and 3 were infected with Plasmodium knowlesi (W_1 strain) by inoculating 1x106 parasitized RBC intravenously. The thick and thin blood smears stained with Giemsa stain were observed for recording parasitaemia. The three monkeys were treated with dfferent doses of mefloquine (80, 40 and 20mg/kgx3 doses) by oral route.

Monkey No. 1:

On day 3 of infection when the parasitaemia was approximately 0.7% a dose of 80mg/kg mefloquine hydrochloride was administered for 3 consecutive days. The monkey was parasite negative after the second dose. The parasitaemia showed recrudescence 55 days after the third dose of mefloquine. The parasitaemia rose to 2.5 and 8.0% on day 58 and 60 respectively. (Fig.9). On day 60 the monkey was treated with 7.5mg/kg chloroquine (base) orally for 3 successive days with a view to determine the sensitivity of the parasite to chloroquine. The monkey remained negative after chloroquine treatment till follow-up of 40 days. The parent line resistant to 80mg/kg dose of mefloquine has been cryopreserved for resistance reversal study with penfluoridol.

Monkey No. 2:

When the initial parasitaemia was 0.5%; the monkey was treated orally with 40mg/kg mefloquine hydrochloride for 3 consecutive days. The parasitaemia became -ve after the second dose, but there was recrudescence on day 11 after the last dose of mefloquine. This monkey was again treated with 40mg/kg mefloquine orally for 3 consecutive days and was cured (Fig. 8).

Monkey No. 3:

The 3rd monkey with 0.3% parasitaemia, was treated with 20mg/kg mefloquine hydrochloride

orally for 3 consecutive days. The monkey was negative after the second dose. There was recrudescence on day 9 of the last dose of mefloquine. The monkey was again treated with 20mg/kg mefloquine orally for 3 consecutive days. The monkey showed absence of parasitaemia after the second dose. On day 8 after the last dose, the monkey showed recrudescence. 20mg/kg mefloquine was again administered orally for 3 consecutive days. The monkey was cured after the third dose of mefloquine but there was recrudescence and the parasitaemia reached 1.4% on day 14 after the last dose of mefloquine. The parasitized RBC were preserved in liquid nitrogen. The monkey was cured with 7.5mg/kg chloroquine base x3 day orally.

9. DRUG RESISTANT RODENT MALARIA STRAINS:

(i) The following drug resistant lines of rodent malaria parasite **P. berghei** have been cryopreserved.

1. Chloroquine resistant strain (upto 128mg/kgx4
2. Mefloquine resistant strain (upto 128mg/kgx4)
3. Quinine resistant strain (upto 400mg/kgx4)

(ii) A multiple resistant strain of P. yoelii nigeriensis resistant to chloroquine (128mg/kgx4), mefloquine (128mg/kgx4) and quinine (400mg/kgx4) has been cryopreserved.

10. STUDIES ON REVERSAL OF DRUG RESISTANCE:

A large number of reports have appeared in literature during the last decade in which the chloroquine resistance of the cultured drug resistant isolates of P. falciparum had been claimed to be reversible in vitro by certain agents/compounds designated as reversal agents/resistance modulators/MRD modifiers. According to available reports from literature, in presence of resistance reversal agents a much lower dose of chloroquine is required to kill the resistant P. falciparum in

culture. Sofar, very few studies on drug resistance reversal have been carried out in the in vivo models. But the published data do not prove conclusively that available resistance reversal agents would be potentially safe clinically and effective. Emphasis would be, therefore, continued to establish chloroquine/mefloquine resistant P. knowlesi models to evaluate these claims and also to complete preclinical studies on a few selected reversal agents, which could be identified as potential candidate compounds for clinical trials.

DRUG RESISTANCE REVERSAL STUDIES AGAINST MULTI RESISTANT P. YOELII NIGERIENSIS:

This strain is resistant to chloroquine (128mg/kgx4), mefloquine (128mg/kgx4) and also quinine (400mg/kgx4), and it is 100% lethal for swiss mice.

VERAPAMIL:

Verapamil which is a calcium channel blocker has been evaluated for chloroquine resistant reversal activity against multiresistant P. yoelii nigeriensis. Two drug administration schedules from day 0-3 and day 3-6 post infection were used.

(i) Day 0-3 treatment:

Chloroquine alone was given at 8mg/kg dose, verapamil at 25mg/kg. Besides a combination of verapamil 10 and 25mg/kg with 8mg/kg dose of chloroquine was tested (Table-22). Mean survival time (MST) of verapamil and chloroquine combination was slightly extended (12.25-12.63 days) in comparison to MST of 10.75 days observed in chloroquine control group. Extension of MST was observed only at higher doses of verapamil (10 and 25mg/kg) and no extension of MST was observed with lower dose of verapamil (0.5 and 1.0mg/kg).

(ii) Day 3-6 treatment:

In this second group, the drug administration schedule was from day 3-6 post infection.

Chloroquine treated group of mice showed MST 21.14 days whereas different doses of verapamil with 8mg/kg chloroquine showed mean survival time ranging from 15.17, 23.67, 24.60 to 25.57 days and the increase in MST was directly related to the increasing dose of verapamil from 5-25 mg/kg (Table-23). The study shows a limited reversal effect of verapamil when given with chloroquine. It may be pointed out that the number of animals surviving with combination of verapamil and chloroquine has not been consistent in different experiments.

NIFEDIPINE:

This drug was also tested in combination with chloroquine against multi-drug resistant P.yoelii nigeriensis using 3-7 day post infection treatment schedule. Before drug treatment the parasitaemia was 0.5%. In groups given nifedipine + 8 mg/kg chloroquine, the maximum survival time was 24.7 and 24.8 days in comparis n to chloroquine alone which gave 21.14 days (Table-24). In conclusion the nifedipine has provided marginal extension of MST, specially at the high dose.

EVALUATION OF WR 238605 FOR CHLOROQUINE RESISTANCE REVERSAL ACTION:

For resistance reversal studies with WR 238605 chloroquine resistance strain of P. yoelii nigeriensis was used. Chloroquine treatment (4.0 and 8.0 mg/kg x4 days) resulted in MST of 12.8+4.2 and 17.8+9.4 days respectively, while chloroquine at 4.0 and 8.0 mg/kg when given together with 0.5mg/kg of WR 238605, resulted in only slight extension of MST from 12.8+4.2 to 14.4+5.9 at 4.0mg chloroquine dose, and from 17.8+9.4 days to 19.4+9.0 days at 8.0 mg chloroquine dose. Administration of WR 238605 (0.5mg/kg) with chloroquine(4.0 or 8.0 ;mg/kg) extended the MST by nearly 2 days at both the dose levels of chloroquine used in the study (Table-25).

The marginal extension of MST when WR 238605 is administered with chloroquine suggests some additive effect of the drug combination specially when both the drugs are blood schizontocides.

EVALUATION OF WR 238605 FOR MEFLOQUINE RESISTANCE REVERSAL ACTION:

Resistance reversal effect of WR 238605 (0.5mg/kg dose) alone and in combination with various doses of mefloquine (1.0, 2.0, 4.0 and 8.0mg/kg x4 days) was evaluated using multi resistant P. yoelii nigeriensis. This rodent parasite is resistant to mefloquine at 128mg/kgx4 days schedule. WR 238605 (0.5mg/kg) alone did not extend the mean survival time of the mice which was 6.2 days compared to 5.8 days in control group (Table-26). Mefloquine alone (1.0-8.0mg/kg doses) produced gradual increase of MST from 6.6 days to 15.0 days corresponding to increasing dose levels of mefloquine. When mefloquine doses (1.0, 2.0, 4.0 and 8.0 mg/kg) were given together with fixed dose of WR 238605 (0.5mg/kg) there was no increase in MST which varied from 6.6, 10.0, 11.0 to 13.2 days respectively corresponding with the increasing dose level of mefloquine. The study shows no significant resistance reversal effect of WR 238605 against mefloquine resistant strain of parasite.

3. WRAIR CDRI COLLABORATIVE PROJECT

PROGRAMME FOR 1995-96

I. ESTABLISHMENT OF IN VIVO MODEL FOR RODENT RESISTANCE REVERSAL STUDIES:

A large number of agents are reported in literature which have been shown to reverse chloroquine resistance of cultured P. falciparum. Resistance reversal potential of WR 238605. WR 242511, WR 268954, Verapamil, Nifidepin, Penfluoridol, Desipramine etc. against chloroquine, mefloquine and halofantrine resistant rodent parasites will be studied.

II. SELECTION OFR DRUG RESISTANT SIMIAN PARASITES

Specially chloroquine/mefloquine resistant strains of P.knowlesi will be used for resistant reversal studies with a few potent reversal agents in rhesus monkeys. This is a high priority area for

both WRAIR and CDRI programmes.

III. P. FALCIPARUM:

Semi automated antimalrial screen using H hypoxanthine incorporation to be established using standard antimalarials to generate base line data.

IV. HEPATOMA

Hep G2 cell line infection with sporozoites of P. berghsei NK 65 to expand tissue stage screening.

V. TO ESTABLISH PRIMARY RHESUS HEPATOCYTE CULTURE:

Sporozoites of P. cynomolgi B infectivity of the cell culture will be given priority to establish in vitro screening of tissue schizontocides.

VI. ALTERNATE TREATMENT REGIMENS FOR CONTROL OF CHLOROQUINE RESISTANT P. VIVAX

Due to the recent emergence of chloroquine resistant strains of P.vivax, there is need to identify alternate antimalrials drugs to be used as blood schizontocide in combination with primaquine or WR 238605. Studies have been planned to conduct final re-validation of the efficacy of the following combinations against both blood induced P. cynomolgi B in rhelsus monkeys, as well as in anti-relapse test.

1. Halofantrine plus WR 238605.
2. Mefloquine plus WR 238605.

VII. IN VITRO MODEL FOR MET-HB TOXICITY OF 8-AMINOQUINOLINES

VIII. MOLECULAR PROBE FOR DRUG RESISTANT FALCIPARUM MALARIA

Probe for identification of chloroquine resistant isolates will be procured for WRAIR if available and drug resistant strains of P. falciparum from India wil be screened. Identificastion of chloroquine resistant P. falciparum strains will receive high priority, if suitable probe is available from WRAIR, WASHINGTON or AFRAMIS, Bengkok. This work will be taken up.

IX. VACCINATION; RECOMBINANT HUMAN INTERLEUKIN-12 (r Hu IL-12) PROTECTION FROM MALARIA IN RHESUS MONKEYS. (SPONSORED BY U.S.NAVAL MEDICAL RESEARCH INSTITUTE, COLLABORATION WITH DR. J.M.CRETCHER, U.S. NAVY).

EXPERIMENTAL PROCEDURES:

Course of P.cynomolgi infection in rhesus monkeys: Interavenous injection of P.cynomolgi sporozoites results in universal blood stage infection about 10 days later (range 7-30 days). P. cynomolgi is a relapsing malaria, similar to human vivax malaria. Relapses generally occur 10-15 days after clearance of blood-stage infection. In spleen intact animals, parasitaemia ranges from 3-8%. Parasitemias are approximately twice as high in splenectomized animals. The infection in rhesus monkeys is generally self-limited and the monkeys exhibit no overt distress; they eat and drink normally at all levels of parasitaemia. Mortality does occur, generally in splenectomized animals with high parasitaemias. Analgesics are generally not required. Infected animals can be cured with chloroquine 5 mg/kg and primaquine 1mg/kg for 7 days. Rhesus monkeys weigh approximately 5 kg.

(1) Determination of protective dose and schedule of IL-12:

Four groups of 4 monkeys each will be tested. The formulation will be delivered intramuscularly (25 g needle) in the quadriceps muscle of the leg. rHuIL-12 will be diluted in sterile 1% normal monkey serum in PBS (pH 7.2) to give required doses of rHuIL-12 in 0.5 ml. Control monkeys will be given 0.5 ml. 1% monkey serum in PBS (pH 7.2). rHuIL-12 will be given in the following regimens:

GROUP	IL-12 DOSE	TREATMENT DURATION	NO. OF MONKEYS
1.	100ng/kg	Day -2 to +10(alternate day)	4
2.	1μg/kg	Day -2 to +10 (alternate day)	4
3.	20μg/kg	Day -2 (single dose)	4
4.	Control	Day -2 to +10(alternate day)	4

If found to be efficacious, an additional experiment consisting of one study group (3 monkeys) and one control group (3 monkeys) will be performed to verify positive results. The above dose and regimen found to be most protective will be used. If similar results are obtained with more than one group then the lower dose, or single dose, will be used.

Testing to date has proven rHUIL-12 safe in monkeys. The above doses were recommended by researchers of Hoffman-LaROche who have performed several rHuIL-12 studies in both rhesus and Siamiri sciurus monkeys. In Siamiri monkeys the above doses were bioactive and safe, with no clinical abnormalities or serious toxicity. Hematologic and serum chemistry abnormalities included mild to moderate anemia and leukocytosis, hypoproteinemia, hypoalbuminemia, hypophosphatemia, and hypocalcemia. Their findings suggest that the above doses should be active and not cause serious adverse effects. Our work in mice with higher IL-12 per weight doses did not show adverse effects.

On day 0 monkeys will be injected in the mid-saphanous vein (using a 25 g needle and 3 ml syringe) with 10,000 sporozoites which have been dissected from the salivary glands of Anopheles stephensi mosquitoes which have been fed on monkeys infected with P.cynomolgi. Beginning on day 7 after infection and continuing for 8 weeks, monkeys will be bled from the marginal ear vein (approximately 10 μl by sterile lancet skin prick) to assess parasitemia by Geimsa-stained blood smear. Smears will be performed daily for the first 3 weeks and twice per week for an additional 5 weeks. Obtaining the blood sample does not require anesthesia and lasts less than one minute. Any discomfort felt by the animal is transitory. Prior to puncture the skin will be swabbed with alcohol. All monkeys that develop parasitaemia willbe cured with chloroquine 5mg/kg and primaquine 1mg/kg for 7 days by oral catheter, except for the 4 control monkeys who will be uysed in Experiment 2 and 3 as discussed below. At the conclusion of the study monkeys will be either returned to the colony for further experiments or euthanized if unsuitable for further studies, according to protocol at the Central drug Research Institute (CDRI).

Human rIL-12 will be used in this sltudy. It will be produced in E.coli and provided by F. Hoffman-LaRoche, Nutley, NJ. An every other day dose is used because of the prolonged half-life of rHuIL-12 in monkeys (14 hours) compared to mouse IL-12 in mice (3 hours).

Determination of IFN-γ levels after rHuIL-12 injection:

As discussed above, the parasite killing effect of IL-12 appears to be mediated by IFN-γ although this has not been assessed in the monkey model. We plan to determine IFN-γ levels following rHuIL-12 administration in order to assess this relationship. Approximately ten 3 ml. blood samples will be obtained from each monkey for determination of IFN-γ levels; this will include a baseline sample prior to rHuIL-12 injection, alternate day samples from day 0 to day 10, and twice weekly samples from day 11 to day 25. This will allow us not only to assess the association of IFN-γ levels with protection but also to determine the kinetics of IFN-γ following rHuIL-12 in the monkey model. Blood will be drawn from the external saphenous vein of the leg using a 25 g needle and 5 ml. syringe. The skin will be swabbed with alcohol prior to venipuncture. Sera will be separated and frozen for later transport to Dr. Ansari in Atlanta for testing.

(2) Determination of effect of rHuIL-12 on infectivity of gametocytes:

Previous work at CDRI in the rhesus P.cynomolgi model has shown that IFN-γ inhibits the ability of gametocytes to infect mosquitoes. This is determined by monitoring the number of oocysts which form on the gut wull of mosquitoes after feeding on gametocytemic monkeys. Gametocytes normally appear about 2 weeks after infection and mosquito oocyst counts over the following ten days range from 20-200. IFN-γ given during the gametocytemic phase has resulted in the complete absence of oocysts in mosquitoes. (Dr. R. Tripathi, CDRI, personal communication). We plan to test the hypothesis that rHuIL-12 will have the same effect due to its ability to stimulate IFN -γ production. The four monkeys used as controls in Experiment 1 will be used for the experiment. About 7 days after the monkeys become gametocytemic from the sporozoite challenge they had previously received (a time of peak gametocytemia), 2 monkeys then will be treated with rHuIL-12 (20 μg/kg in a single dose) and 2 will serve as controls and receive 1% monkey serum as above. Three to four day old A.stephensi mosquitoes will then be fed on the monkeys at 6,24, and 48 hours after treatment. These mosquitoes will be maintained in the insectory and midgut dissections performed on day 7 or 8 to monitor oocyst number.

(3) Determination of effect of rHuIL-12 on asexual blood stages:

Although IL-12 has been shown to have no effect on the asexual blood stages of malaria parasites in the mouse model, this has not been tested in monkeys. We plan to evaluate the effect of rHuIL-12 on asexual blood stages in the rhesus monkeys by monitoring the level of asexual parasites after rHuIL-12 injection in the monkeys used in Experiment 2. Daily blood smears will be obtained for 14 days for comparison of parasitaemia levels in study and control monkeys.

Tabular Data and Figures

TABLE-1

Taole: Serial cyclic passages of sporozoite induced P.cynomolgi B in rhesus monkeys since March,1993.

Sporozoite passage	Date of inoculation	Monkey No.	Sporozoite inoculum i.v.	Day of patency
87	6.3.93	7666	$1.44X10^6$	8
88	13.4.93	7679	$0.73X10^6$	9
89	20.5.93	7680	$1.64x10^6$	8
90	26.6.93	7775	$1.24X10^6$	8
91	5.8.93	7782	$0.70X10^6$	9
92	8.10.93	7827	$0.76x10^6$	9
93	1.12.93	7850	$1.14X10^6$	8
94	10.1.94	7831	$0.96X10^6$	8
95	14.2.94	7911	$1.54X10^6$	8
96	18.3.94	8018	$0.83X10^6$	9

Table 2 Serial cyclic passages of sporozoite induced P.cynomolgi B in rhesus monkeys since March,1994.

Sporozoite passage	Date of inoculation	Monkey No.	Sporozoite inoculum	Day of patency
97	29.4.94	8029	1.14×10^6	8
98	17.6.94	8084	1.40×10^6	8
99	5.8.94	8086	0.72×10^6	9
100	10.9.94	8179	1.15×10^6	8
101	28.10.94	8258	0.86×10^6	9
102	22.12.94	8240	1.24×10^6	8

Table- 3

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 1544 Chloroquine

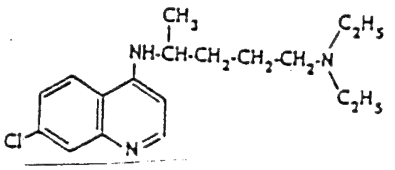
BN: AU 29291

DATE REC'D: Oct.1993

QUANTITIY: 500 gm

VEHICLE: Aqueous

ROUTE Oral



Mol.Wt.= 518

Base= 320

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
3.00 mg/kg	7915	Cured
3.00 mg/kg	7917	Cured

Table-4

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Chloroquine (3 dose regimen)

BN: AU 29291

DATE REC'D: Oct.1993

QUANTITIY: 500 gm

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

BLOOD SCHIZONTOCIDAL TEST (X 3 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
5.0	8083	Recrudescence on day 16
5.0	8088	Recrudescence on day 14
7.5	8035	Cured
7.5	8074	Recrudescence on day 18
10.0	8078	Cured
10.0	7992	Cured
7.5*	8083	Cured
7.5*	8088	Cured
10.0*	8074	Cured

* Monkeys retreated after recrudescence at the lower dose.

TABLE- 5

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose validation in Sp. passage 90)

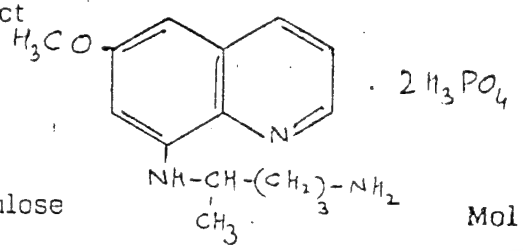
BN: Sigma Product

DATE REC'D:

QUANTITIY:

VEHICLE: Methyl Cellulose

ROUTE Oral



Mol.Wt.= 455

Base= 259

PROPHYLACTIC TEST (X 3 day)

DOSE mg/kg(base)	MONKEY NO. -	RESULT
1,78	7772	Cured
1.78	7776	Cured
Control		
-	7775	Patent on day 8
-	7773	Patent on day 9

TABLE- 6

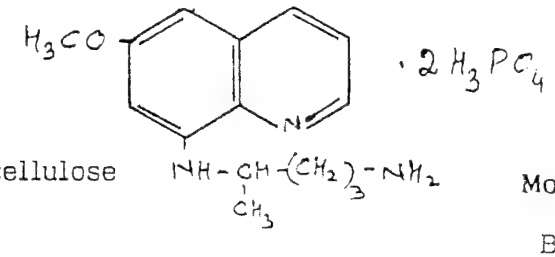
CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose revalidation in serial Sp. Passage 86)

BN: Sigma Product

DATE REC'D:

QUANTITIY:



VEHICLE: Methyl cellulose

Mol.Wt.= 455

ROUTE Oral

Base= 259

RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO. -	RESULT
1.00	7552	Cured
1.00	7556	Cured
0.316	7548	Relapse on day 29
0.316	7560	Relapse on day 37
Chloroquine Control		
-	7558	Relapse on day 16
-	7578	Relapse on day 19

TABLE- 7

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: Primaquine (Dose revalidation in serial Sp Passage 90)

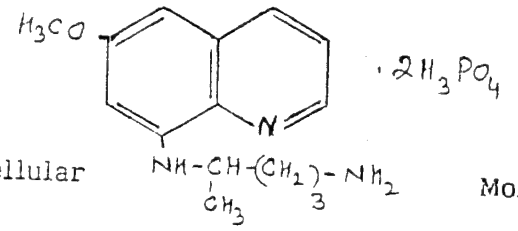
BN: Sigma Product

DATE REC'D:

QUANTITIY:

VEHICLE: Methyl Cellular

ROUTE Oral



Mol.Wt.= 455

Base= 259

RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1.00	7773	Cured
1.00	7774	Cured
Chloroquine Control	7768	Relapse on day 9

TABLE- 8

Comparison of the blood schizontocidal activity of WR 238605 and primaquine against trophozoite induced *P.cynomolgi* B and *P. fragile* in rhesus monkeys.

Dose mg(base)/ kgx7 days	Total course dose mg(base)/kg	No. of monkeys treated	Response to treatment	
			No. of monkeys cured	No.of monkeys showing recrudescence(on day)
A. *Plasmodium cynomolgi* B Infection				
WR238605				
0.316	2.21	4	0	4 (7,13,18,20)
1.00	7.00	12	10	2 (20,26)
3.16	22.12	6	6	0
Primaquine				
1.00	7.00	2	0	2 (10,12)
3.16	22.12	4	0	4 (13,15,16,19)
10.00	70.00	4	1	3 (15,24,28)
B. *Plasmodium fragile* infection				
WR 238605				
0.316	2.21	4	0	4 (16,19,24,28)
1.00	7.0	11	10	1 (36)
3.16	22.12	4	4	0
Primaquine				
1.00	7.00	2	0	2 (13,16)
3.16	22.12	3	1	2 (17,20)
10.00	70.00	3	2	1 (18)

TABLE- 9

Gametocytocidal activity of compound WR 238605 in the P. cynomolgi - A. stephensi - rhesus monkey model

DOSE (Mg/Kg) AT 0 Hr.	TIME OF MOSQUITO FEEDING	PARASITAEMIA/MM^3 ASEXUAL	PARASITAEMIA/MM^3 GAMETO-CYTAEMIA	DAY 7 OOCYST RECORD NO. OF MOSQUITO INFECTED/ DISSECTED (% INFECTI-VITY)	DAY 7 OOCYST RECORD OOCYST NUMBER (MEAN± SD)
1.00	-1Hr.	23112	749	32/37 (86.49%)	33.16±22.18
	+6Hr.	-	-	31/36 (86.11%)	42.26±23.76
	+24Hr.	26215	533	31/39 (79.49)	25.77±17.63
	+48Hr.	7383	321	0/30 (Nil)	-
1.00	-1Hr.	39055	1391	32/40 (80.0)	17.13±10.01
	+6Hr.	-	-	32/44 (72.73%)	13.69±7.20
	+24Hr.	28248	321	0/31 (Nil)	-
	+48Hr.	5992	107	0/23 (Nil)	-
1.00	-1Hr.	48384	6832	25/34 (73.53%)	32.12±13.62
	+6Hr.	-	-	32/40 (80.0%)	31.06±12.73
	+24Hr.	42448	3256	36/48 (75.0%)	30.86±13.81
	+48Hr.	20832	1008	0/25 (Nil)	-
2.00	-1Hr.	54805	2938	43/53 (81.13%)	28.91±18.43
	+6Hr.	-	-	47/58 (81.03%)	27.13±16.30
	+24Hr.	26555	1243	0/38 (Nil)	-
2.00	-1Hr.	42619	2398	25/39 (64.10%)	18.40±10.19
	+6Hr.	-	-	30/48 (62.5%)	17.83±6.80
	+24Hr.	26487	1199	0/36 (Nil)	-
2.00	-1Hr.	42036	3503	22/27 (81.48%)	35.32±13.34
	+6Hr.	-	-	28/34 (82.35%)	26.89±11.19
	+24Hr.	21344	2668	0/31 (Nil)	-
4.00	-1Hr.	73902	3390	28/33 (84.85%)	29.75±12.41
	+6Hr.	-	-	31/36 (86.11%)	38.16±19.28
	+24Hr.	47008	1808	0/30 (Nil)	-

Table-10

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 (Shorter 3 dose regimen)

BN: BK 73252

DATE REC'D: July 1994

QUANTITIY: 10 gm

VEHICLE: Methyl Cellulose Mol.Wt.= [illegible]

ROUTE Oral Base= [illegible]

RADICAL CURATIVE TEST (X 3 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
0.50	8142	Relapse on day 25
0.50	8144	Relapse on day 43
1.00	8076	Cured
1.00	8146	Cured
2.00	8054	Cured
2.00	8116	Cured
-	8077	Relapse on day 30

Monkeys were concurrently administered chloroquine @ 10.0 mg(base)/kg X 3 days.

Table- 11

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 142490 (Mefloquine)

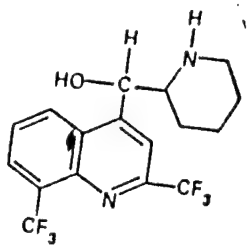
BN: BE. 16387

DATE REC'D: Nov. 1993

QUANTITIY: 1000 mg

VEHICLE: Aqueous

ROUTE Oral



Mol.Wt.= 414.5

Base= 378

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7904	3.16	No parasite clearance
7911	3.16	No parasite clearance
7906	10.0	Cured
7909	10.0	Cured
7903	31.6	Cured
7908	31.6	Cured

Table-12

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Mefloquine

BN: BE 19191

DATE REC'D: July 1994

QUANTITIY: 85 gm

VEHICLE: Aqueous Mol.Wt.= 414.5

ROUTE Oral Base= 378

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
10.0	8140	Cured
10.0	8141	Cured

Table- 13

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMP'D: WR 171669 (Halofantrine)

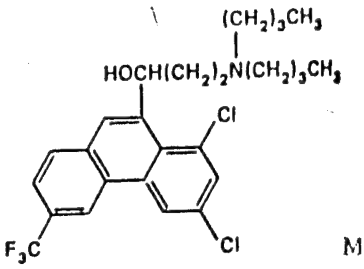
BN: BK 64002

DATE REC'D: Oct. 1988

QUANTITIY: 5 gm

VEHICLE: Aqueous

ROUTE Oral



Mol.Wt.=

Base=

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7912	3.16	Recrudescence on day 12
7921	3.16	Recrudescence on day 14
7919	10.0	Cured
7924	10.0	Cured
7902	31.6	Cured
7905	31.6	Cured

Table-14

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Halofantrine

BN: BK 64002

DATE REC'D: July 1994

QUANTITIY: 50gm

VEHICLE: Aqueous

Mol.Wt.=

ROUTE Oral

Base=

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
10.0	8080	Cured
10.0	8081	Cured

Table-15

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: Halofantrine

BN: BK 64002

DATE REC'D: July 1994

QUANTITIY: 50 gm

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

Expt.II I BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
5.6	8139	Recrudescence on day 19
5.6	8180	Cured
5.6	8265	Cured
5.6	8273	Cured
10.0	8138	Cured
10.0	8181	Cured

Table-16

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 242511

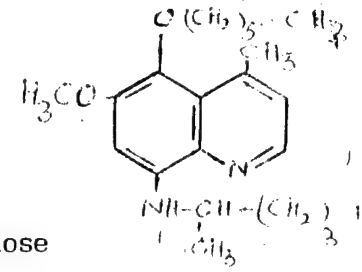
BN: BL 09412

DATE REC'D:

QUANTITIY: 2 gm

VEHICLE: Methyl cellulose

ROUTE Oral



Mol.Wt.= 571

Base= 373

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
7907	0.316	Recrudescence on day 16
7910	0.316	Recrudescence on day 16
7913	1.0	cured
7923	1.0	Cured
7918	3.16	Cured
7922	3.16	Cured

Table-17

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: WR 242511

BN: BL 09412

DATE REC'D:

QUANTITIY: 2 gm

VEHICLE: Methyl Cellulose Mol.Wt.= 511

ROUTE Oral Base= 313

Expt.II BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base)	MONKEY NO.	RESULT
1.0	8075	Cured
1.0	8079	Cured

Table-18

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY

COMPD: **WR 238605 + Halofantrine combination**

BN: BK 73252 , BB 43914

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

BLOOD SCHIZONTOCIDAL TEST (X 7 DAYS)

DOSE mg/kg(base			MONKEY NO.	RESULT
WR 238605 + Halofantrine				
0.316	+	1.00	8264	Recrudescence on day 23
0.316	+	1.00	8275	Recrudescence on day 20
0.316	+	3.16	8274	Cured
0.316	+	3.16	8276	Cured
0.316	+	5.62	8271	Cured
0.316	+	5.62	8272	Cured

Table-19

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 + Halofantrine combination

BN: BK 73252 + BB 43914

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

Expt.1. RADICAL CURATIVE TEST (X 7 day)

DOSE mg/kg(base)	MONKEY NO.	RESULT
WR 238605 + Halofantrine		
0.316 + 3.16	8143	Cured
0.316 + 3.16	8149	Cured
0.316 + 5.62	8145	Cured
0.316 + 5.62	8147	Cured
0.316 + 10.0	8114	Cured
0.316 + 10.0	8115	Cured

Table-20

CDRI PRIMATE ANTIMALARIAL STUDY
PLASMODIUM CYNOMOLGI B RHESUS MONKEY
SPOROZOITE INDUCED TEST

COMPD: WR 238605 + Halofantrine

BN:

DATE REC'D:

QUANTITIY:

VEHICLE: Aqueous Mol.Wt.=

ROUTE Oral Base=

RADICAL CURATIVE TEST (X 7 day)

Expt.II

DOSE mg/kg(base)			MONKEY NO.	RESULT
WR 238605 + Halofantrine				
0.316	+	1.78	8243	Relapse on day 26
0.316	+	1.78	8244	No relapse till day 30
0.316	+	3.16	8238	No relapse till day 30
0.316	+	3.16	8241	No relapse till day 30
0.316	+	5.62	8237	No relapse till day 30
0.316	+	5.62	8242	No relapse till day 30
0.10	+	10.0	8245	Relapse on day 13
0.10	+	10.0	8246	Relapse on day 15
0.316	+	-	8239	Relapse on day 26

Table-21 Sequential maintainence of P.knowlesi for selection of chloroquine resistant strain by interrupted subcurative therapy.

No.	Monkey No..	Date of inoculation	Exposure to chloroquine			Isolate cryopreserved
			No. of doses	Duration (Days)	Total dose (mg/kg)	
Rh-1	7943	19.1.94	5 doses (0.5-3.0 mg/kg)	8 days	7mg/kg	
Rh-2	7945	28.1.94	25 doses (.2-3 mg/kg)	82 days	23.7 mg/kg	R1- 2.4.94 R2- 26.4.94
Rh-3	8027	20.4.94	32 doses (.5-.2 mg/kg)	75 days	24.5 mg/kg	
Rh-4	8095	4.7.94	11 doses (.5-2 mg/kg)	44 days	10 mg/kg	
Rh-5	8087	17.8.94	4 doses (.5-2 mg/kg)	27 days	5.5 mg/kg	R3- 13.9.94
Rh-6	8282*	22.12.94	13 doses (1-2 mg/kg)	54 days (till 14.2.95)	16 mg/kg	

* Monkey inoculated with cryopreserved sample (R3) of 13.9.94.

Table-22:

Effec ts of different concentrations of Verapamil with chloroquine on reversal of drug resistance in P.yoelii nigeriensis strain

Strain: P.yoelii nigeriensis (multi drug resistant), Inoculum : $1x10^6$ parasites; Route: Oral; Treatment: 4 days (i.e. from day 0 - Day +3)

Drug	Dose (day)-+3)	Day 4 Mean±SD±SE	% Parasitaemia Record (Mean±SD) (No. of mice surviving)						
			Day 7	Day 14	Day 18	Day 21	Day 24	Day 28	MST
Control	-	9.38±4.7±1.9 (6)							6.0
Chloroquine	8mg/kg	Nil (8)	3.32±4.5 ±1.6 (8)	7.5±0.0	7.0±0.0				10.75
Verapamil	25mg/kg	18.7±11.6 (7)	32.9±15.9 (4)						8.4
Verapamil + Chloroquine	25mg/kg + + 8mg/kg	Nil (8)	0.3±0.5 ±0.2 (8)	8.6±10.5 ±5.3 (4)	3.3±4.7 ±3.3 (2)	0.2±0.3 ±0.2 (2)	Nil (1)	Nil (1)	12.25
Verapamil + Chloroquine	10mg/kg + + 8mg/kg	Nil (8)	3.7±9.8± ±3.5 (8)	5.2±2.9 ±2.1 (2)	8.0± (1)	0.4± (1)	-	-	12.63
Verapamil + Chloroquine	1.0mg/kg + 8mg/kg	Nil (8)	5.3±7.9 ±2.8 (9)						9.8
Verapamil + Chloroquine	0.5mg/kg + 8mg/kg	Nil (7)	1.4±3.5 ±2.5 (7)						9.13

Table-23.

Effects of different concentration of Verapamil with chloroquine on rversal of drug resistant
Strain: P.yoelii nigeriensis; Inoculum: $7x10^6$; Routeof drugs: Oral, Dose time; 4 days (day 3-6)

Drug	Dosemg/kg (day 3-6)	Day 4	& Parasitaemia (No. of mice surviving) Mean±AS) Day 7	Day 10	Day 14	Day 18	Day 21	Day 24	Day	MST
Control Av. 2.5% of 25 mice		Nil (5)	2.68±0.8 ±0.6 (2)							7.4
Chloroquine	8	Nil (7)	0.3±0.8 ±0.3 (7)	6.3±3.8 ±1.5 (6)	4.65±4.5 ±2.2(4)	1.8±2.8 ±1.6 (4)	0.2±0.2 ±0.1 (4)	Nil (4)	Nil (3)	21.14
Chloroquine	16	Nil (7)	0.4±0.8 ±0.3 (7)	2.9±3.5 ±1.3 (7)	1.37±0.3 ±0.2 (3)	0.2±0.3 ±0.2 (3)	0.1±0.2 ±0.1 (3)	Nil (3)	Nil (3)	19.14
Verapamil	25	- (7)	17.5±8.9 (3)	20.0±0.0 (1)						8.3
Verapamil + Chloroquine	5	Nil (7)	0.14±0.4 ±0.14 (7)	8.5±6.8 ±2.6 (7)	2.8±4.4 ±1.8 (6)	1.23±2.8 ±1.2 (6)	Nil (6)	Nil (6)	(6)	25.75
Verapamil + Chloroquine	8	Nil (30)	Nil (3)	6.4±4.9 ±2.8 (3)	21.7±22.5 ±12.9 (3)	2.8±3.9 ±2.8 (2)	Nil (2)	Nil (2)	Nil (2)	24.60
Verapamil + Chloroquine	8	Nil (6)	Nil (6)	2.2±1.6 ±0.65(6)	4.8±7.3 ±3.3(5)	0.7±1.4 ±0.7(4)	Nil (4)	Nil (4)	Nil (4)	23.67
Verapamil + Chloroquine	8	Nil (6)	0.01±0.02 ±0.01 (7)	4.5±2.8 ±1.13 (6)	13.2±12.0 ±6.9 (3)	1.3±1.8 ±1.3 (2)	Nil (1)	Nil (1)	Nil (1)	15.71

Tabel-24:

Curative efficacy/chloroquine resistant reversal activity of nifedepin with chloroquine.

Drug	Dose	Drug schedule	Parasitaemia% Mean±SE (no. of mice surviving) Day 4	Day 7	Day 14	Day 18	Day 21	Day 28	MST
Nifidepine + Chloroquine	25mg/kg + 8mg/kg	3-7 days	(7)	0.5±0.5 (7)	7.35±1.79 (7)	1.75±1.03 (4)	Nil (3)	Nil (3)	20.9
Nifidepine + Chloroquine	15mg/kg + 8mg/kg	3-7	(7)	0.07 ±0.07	6.27 ±2.61	0.083 ±0.06	Nil (3)	Nil (3)	24.7
Nifidepine + Chloroquine	10mg/kg + 8mg/kg	3-7	(6)	Nil (6)	1.16±1.16 (6)	1.27±1.27 (6)	Nil (3)	Nil (3)	24.8
Nifidepine + Chloroquine	5mg/kg +	3-7	(7)	Nil (7)	2.20±2.20 (3)	5.6±0.0 (1)	-	-	14.4
Nifedepine	25mg/kg	3-7	(7)	36.25 ±13.29 (2)	-	-	-	-	7.1
Chloroquine	8mg/kg	3-7	(7)	0.29 ±0.28 (7)	4.67 ±2.23 (4)	1.8 ±1.60 (4)	0.15 ±0.09 (4)	Nil	21.14
Control	- -	-	(5)	26.8±0.64 (2)					7.4

Table-25:

Evaluation of WR 238605 with chloroquine against *P.yoelii nigeriensis* (Multi Drug Resistant) for Drug Reversal study

Treatment schedule D o→D +3, Route of drug administration (Oral) Av. wt. of mice = 20 gm

Drug	Dose mg/kg	No. of mice	Mean of % parasitaemia on days							M.S.T.
			Day 4	Day 7	Day 10	Day 14	Day 21	Day 28	No. of mice survived	
Control	-	5	25.81 ± 3.2							6.2±.82
Chloroquine + WR 238605	8+0.5	5	0.75 ± .35	1.16 ± .52	1.45 ± .52	2.7 ± .98	-ve	-ve	2	19.4±9.0
Chloroquine + WR 238605	4+0.5	5	0.88 ± .10	.98 ± .20	1.8 ± .75	2.5 ± .70	2.5 ±0			14.4±5.9
Compound + WR 238605	0.5	5	11.33 ±3.2							5.0± .83
Chloroquine	8.0	5	1.48 ±.48	2.0 ±.57	2.25 ±.25	2.55 ±.70	-ve	-ve	2	17.8±9.4
Chloroquine	4.0	5	2.44 ±.72	2.4 ±.73	4.33 ±.28	6.5 ±0				12.8±4.2

Table-26:

Evalaution of mefloquine with WR 238605 for study of drug reversal activity against P.yoelii nigeriensis(MDR)

Treatment schedule- D_0-D+3 Av.wt. of mice= 20 gm

Drug	Dose mg/kg	No.of mice	Mean of % parasitaemia on days				MST
			Day 4	Day 7	Day 10	Day 14	
Mefloquine	1.0	5	3.8	7.0	-	-	6.6
Mefloquine	2.0	5	3.7	4.5	4.5	-	9.2
Mefloquine	4.0	5	0.24	.58	.58	1.3	14.0
Mefloquine	8.0	5	0.24	3.45	.83	1.29	15.0
WR 238605	0.5	5	26.6	-	-	-	6.2
WR 238605+Mefloquine	0.5+1.0	5	1.8	5.0	-	-	6.6
WR 238605+Mefloquine	0.5+2.0	5	1.7	2.25	4.5	5.0	10.0
WR 238605+Mefloquine	0.5+4.0	5	1.06	1.25	1.58	2.2	11.0
WR 238605+Mefloquine	0.5+8.0	5	0.22	.32	.87	1.15	13.2
Control	-	5	27.6	-	-	-	5.8

Fig. -1

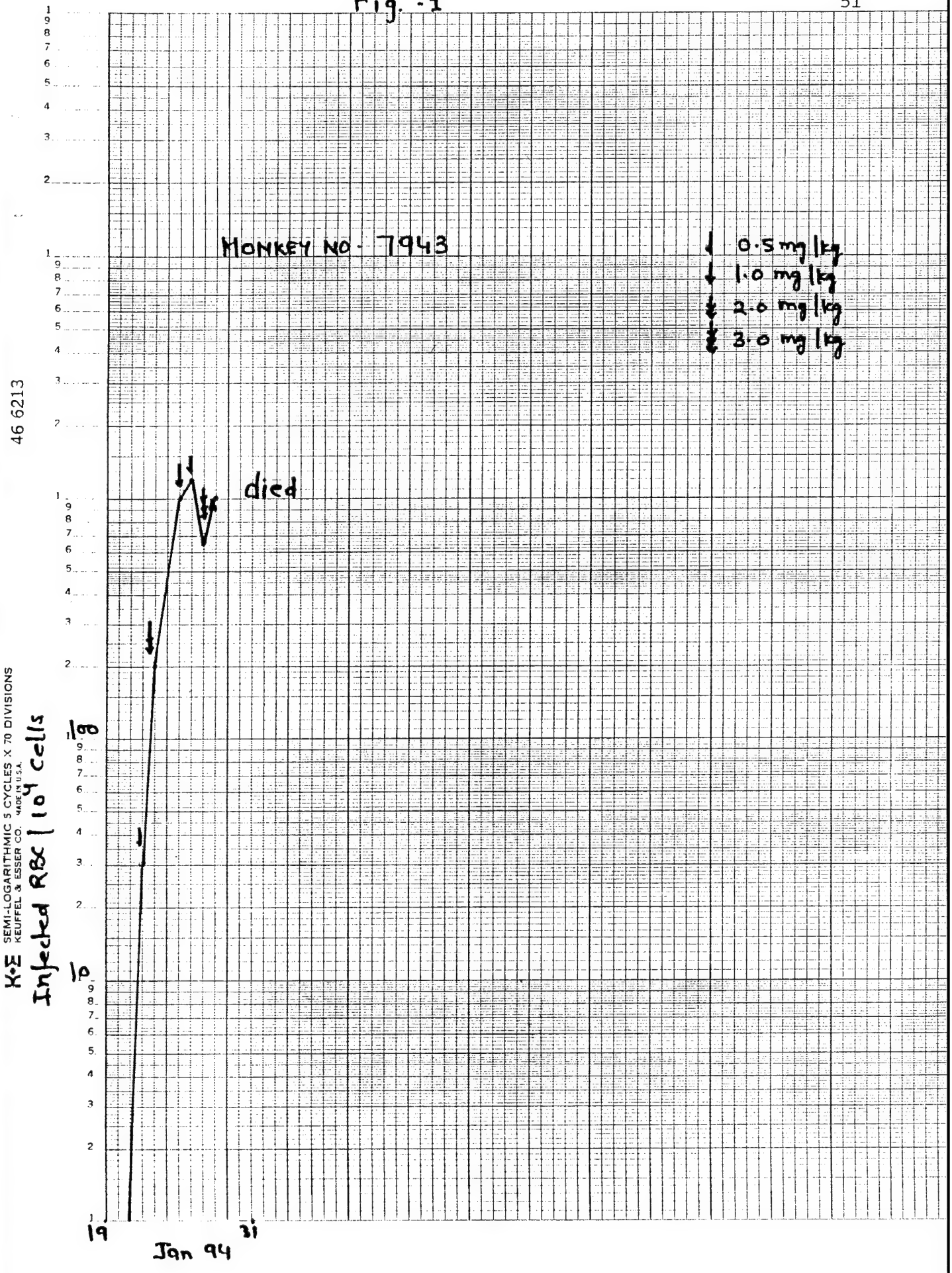


Fig- 2.

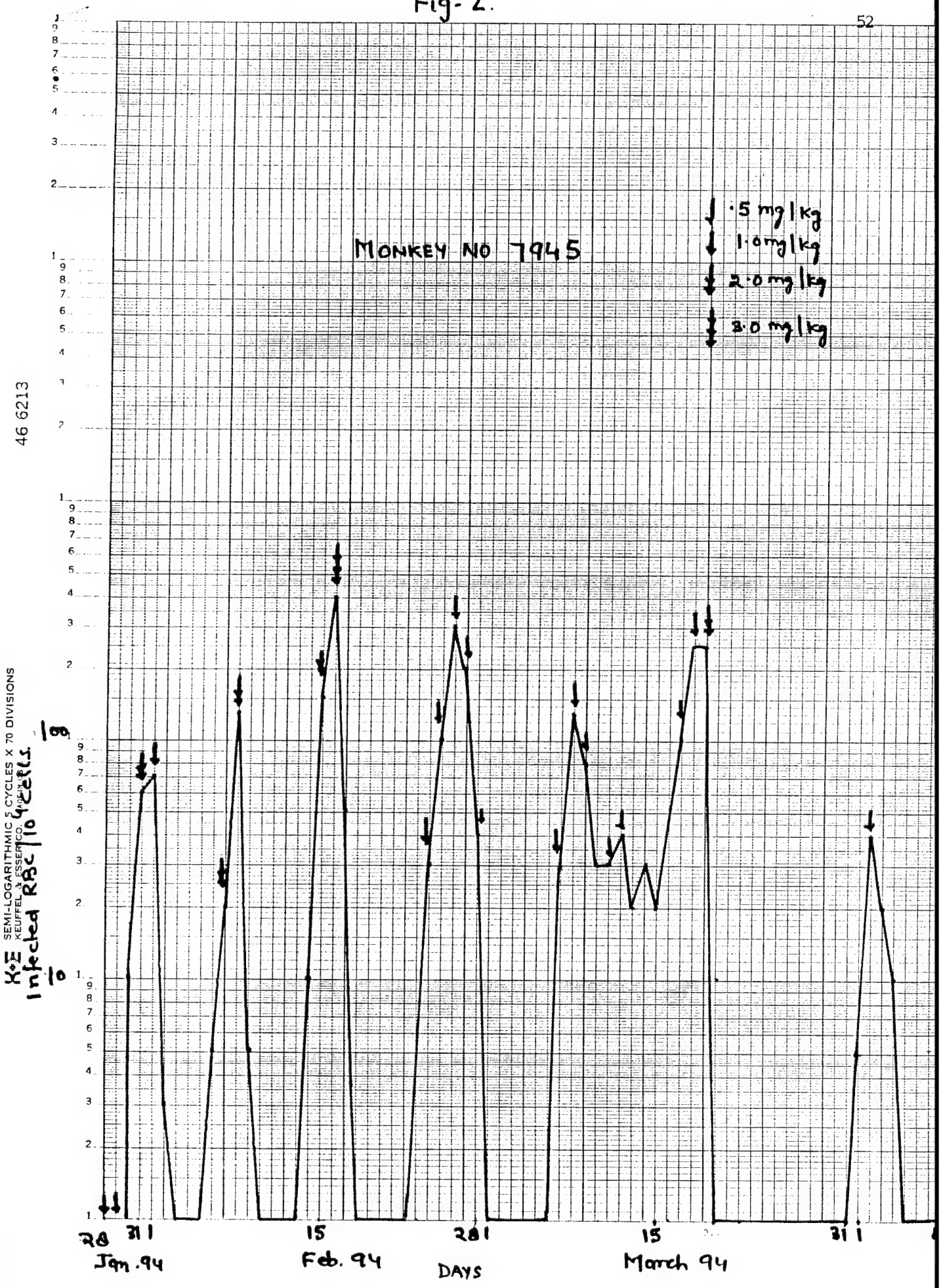

MONKEY NO 7945
·5 mg/kg
1·0 mg/kg
2·0 mg/kg
3·0 mg/kg
46 6213
SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO.
K·E
Infected RBC/10 cells
log
1/10
28
31
15
28
15
31
Jan. 94
Feb. 94
March 94
DAYS

Fig. 2. Contd.

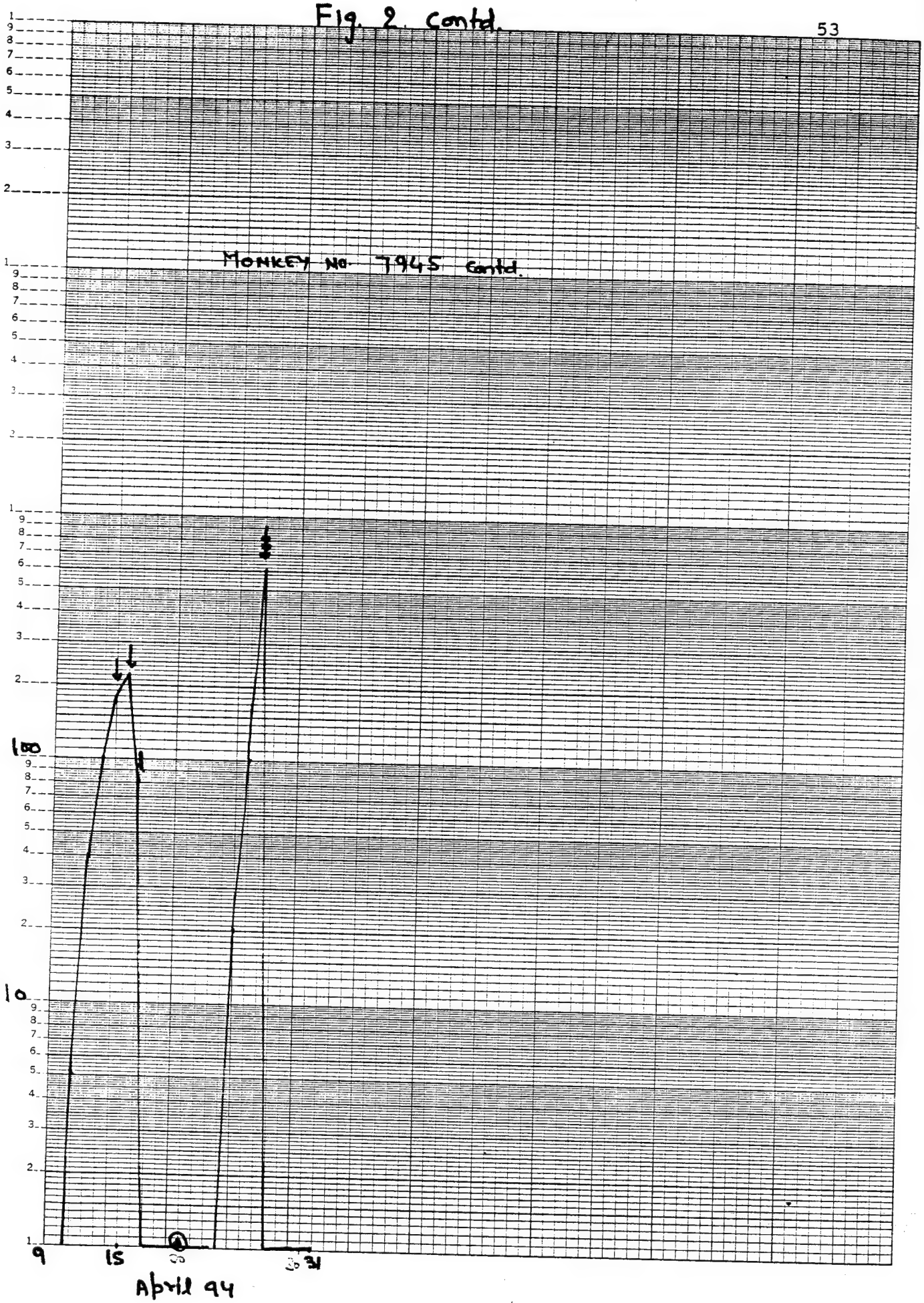

MONKEY No. 7945 Contd.
K&E SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 6213
Infected RBC / 10^4 cells.
100
10
9
15
20
31
April 94

Fig - 3

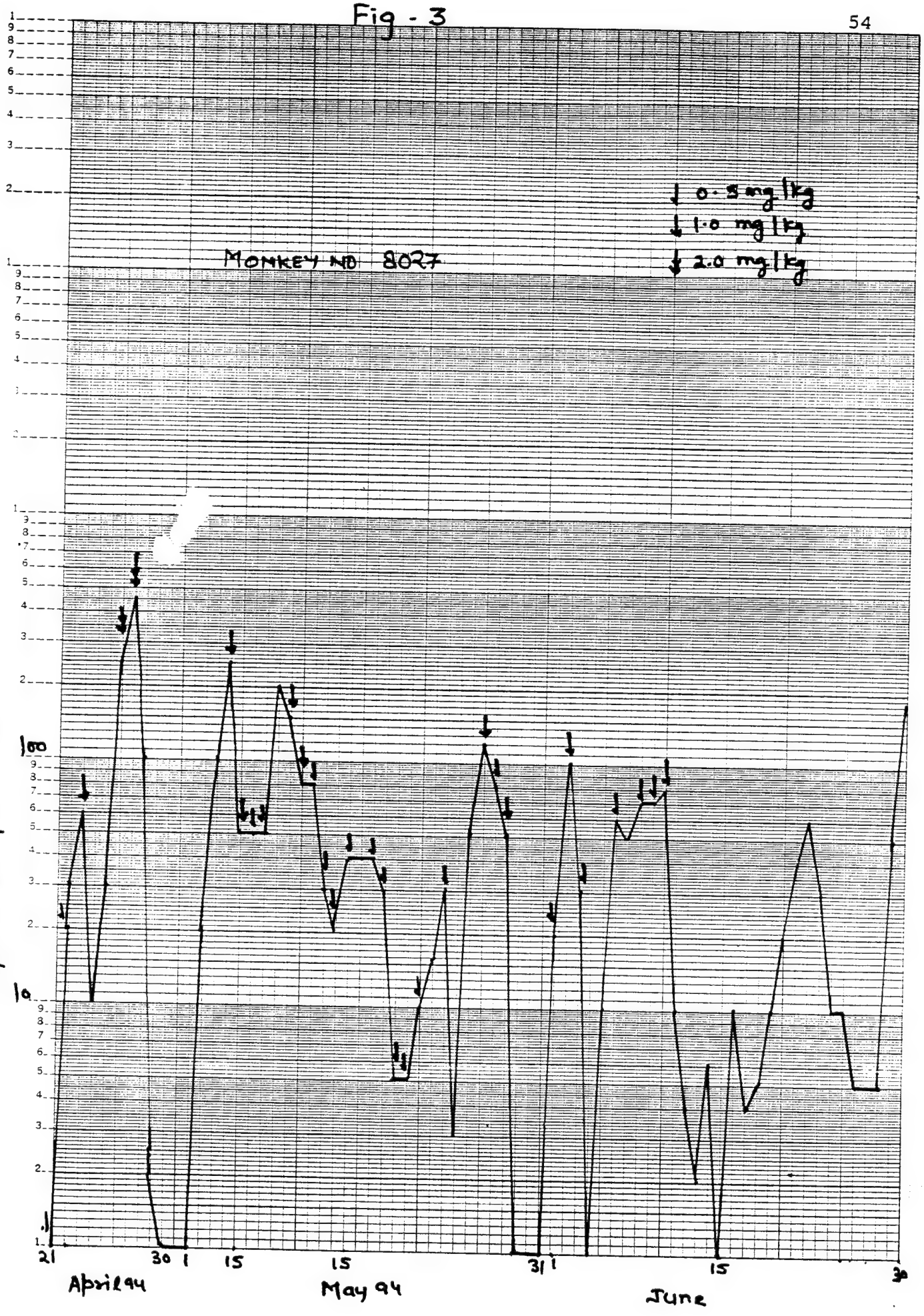


Fig - 3 contd.

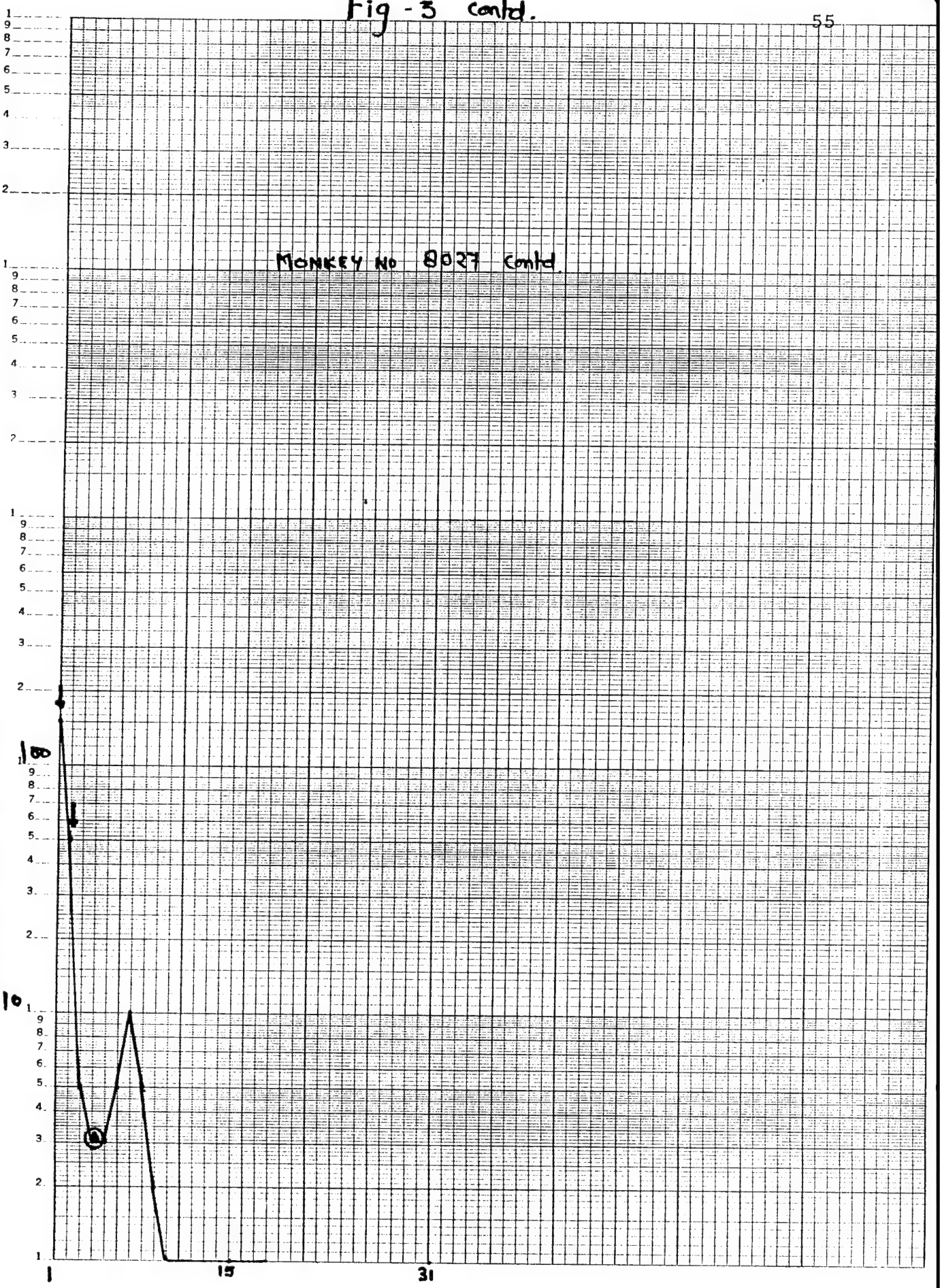


Fig - 4

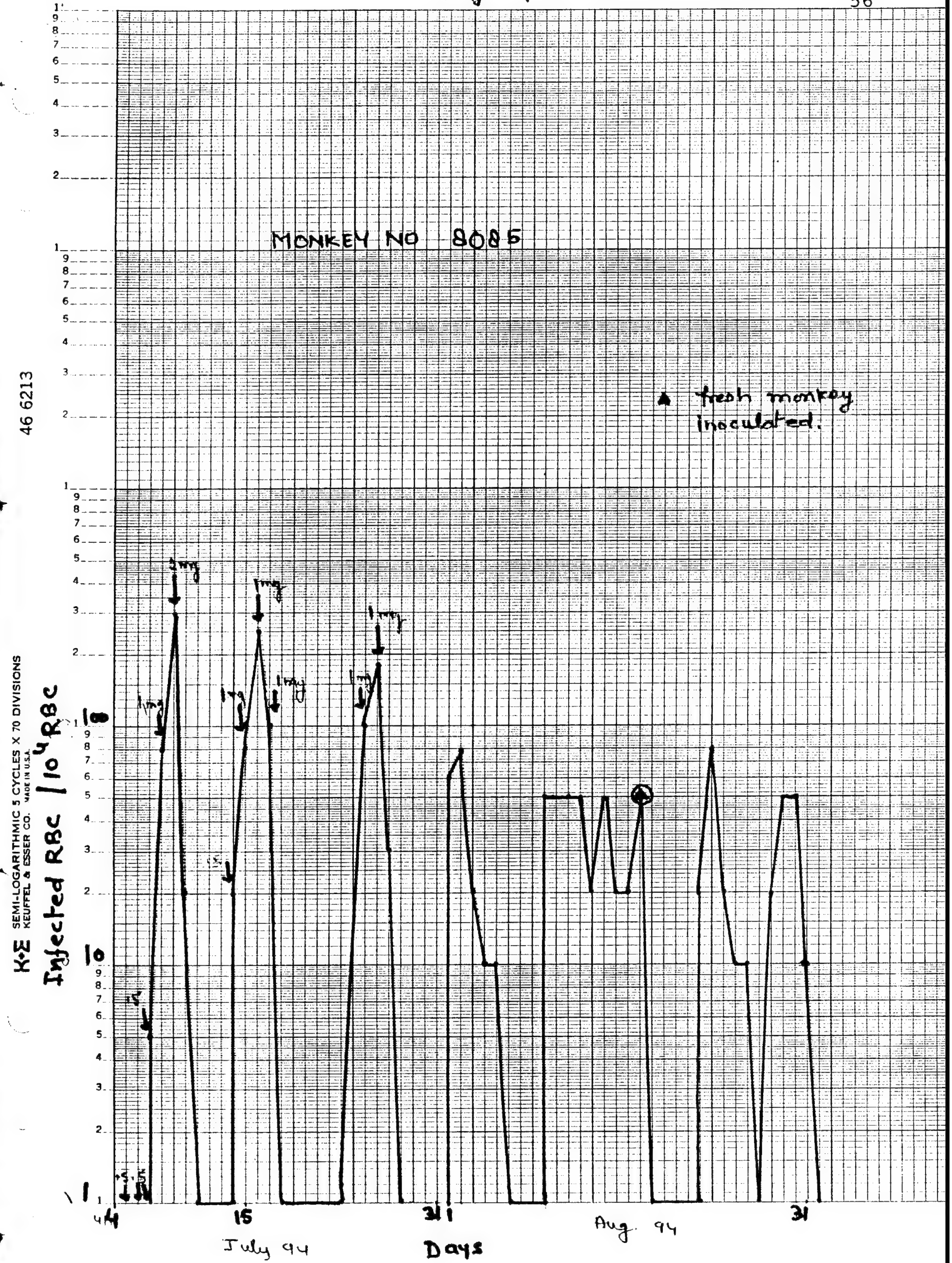
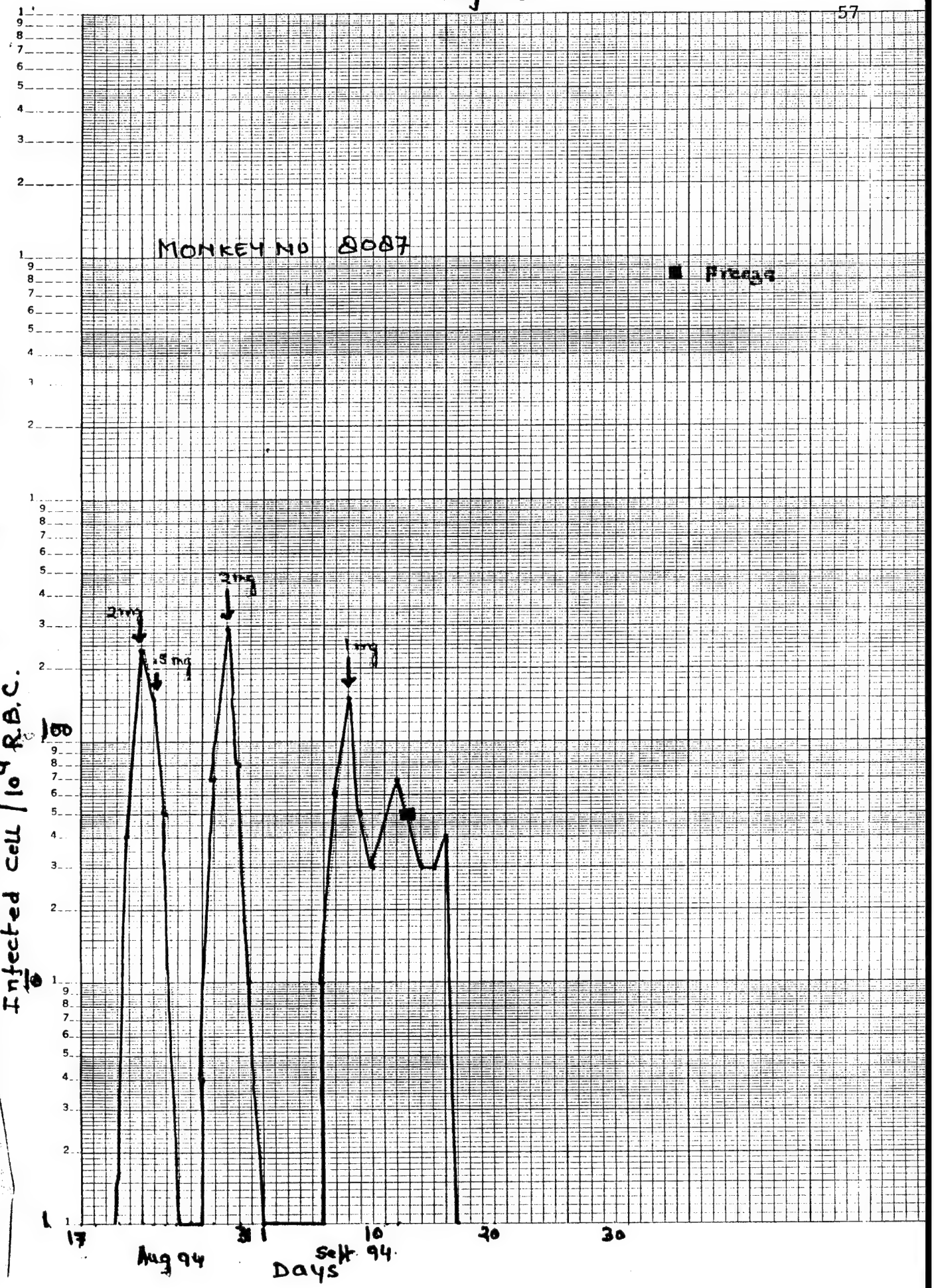

MONKEY NO 8085
▲ fresh monkey inoculated.
Infected RBC / 10^4 RBC
100
10
1
1 mg
15
31
July 94
Aug. 94
Days
K+E SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS
KEUFFEL & ESSER CO. MADE IN U.S.A.
46 6213

Fig - 5


MONKEY NO 8087
■ Freeze
2mg
.5 mg
2mg
1mg
K·E SEMI-LOGARITHMIC 5 CYCLES X 70 DIVISIONS MADE IN U.S.A. KEUFFEL & ESSER CO.
46 6213
Infected Cell / 10⁴ R.B.C.
log
17
Aug 94
31
Sept 94
10
20
30
Days

MONKEY NO 8282

Fig. 6

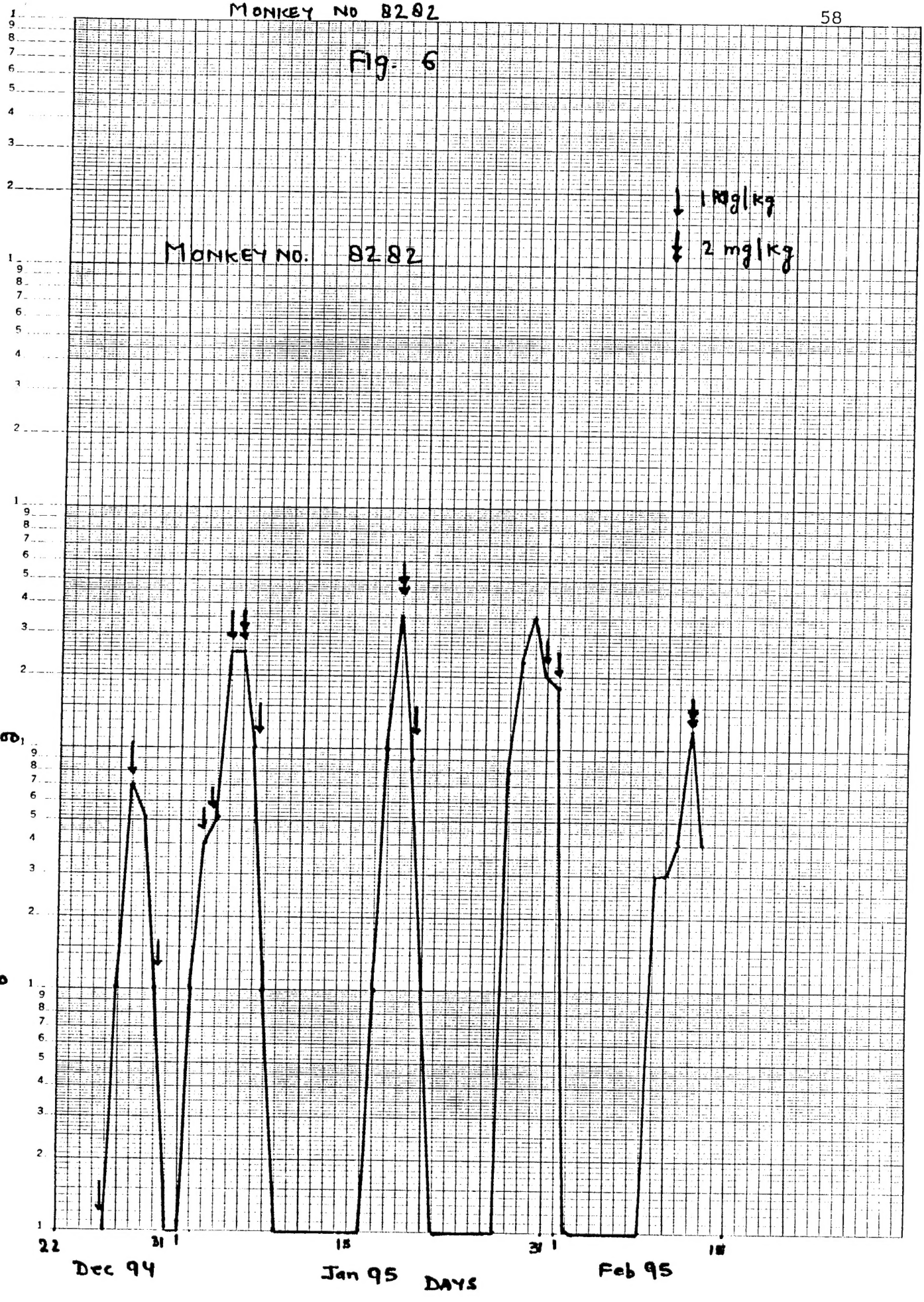


Fig.7.

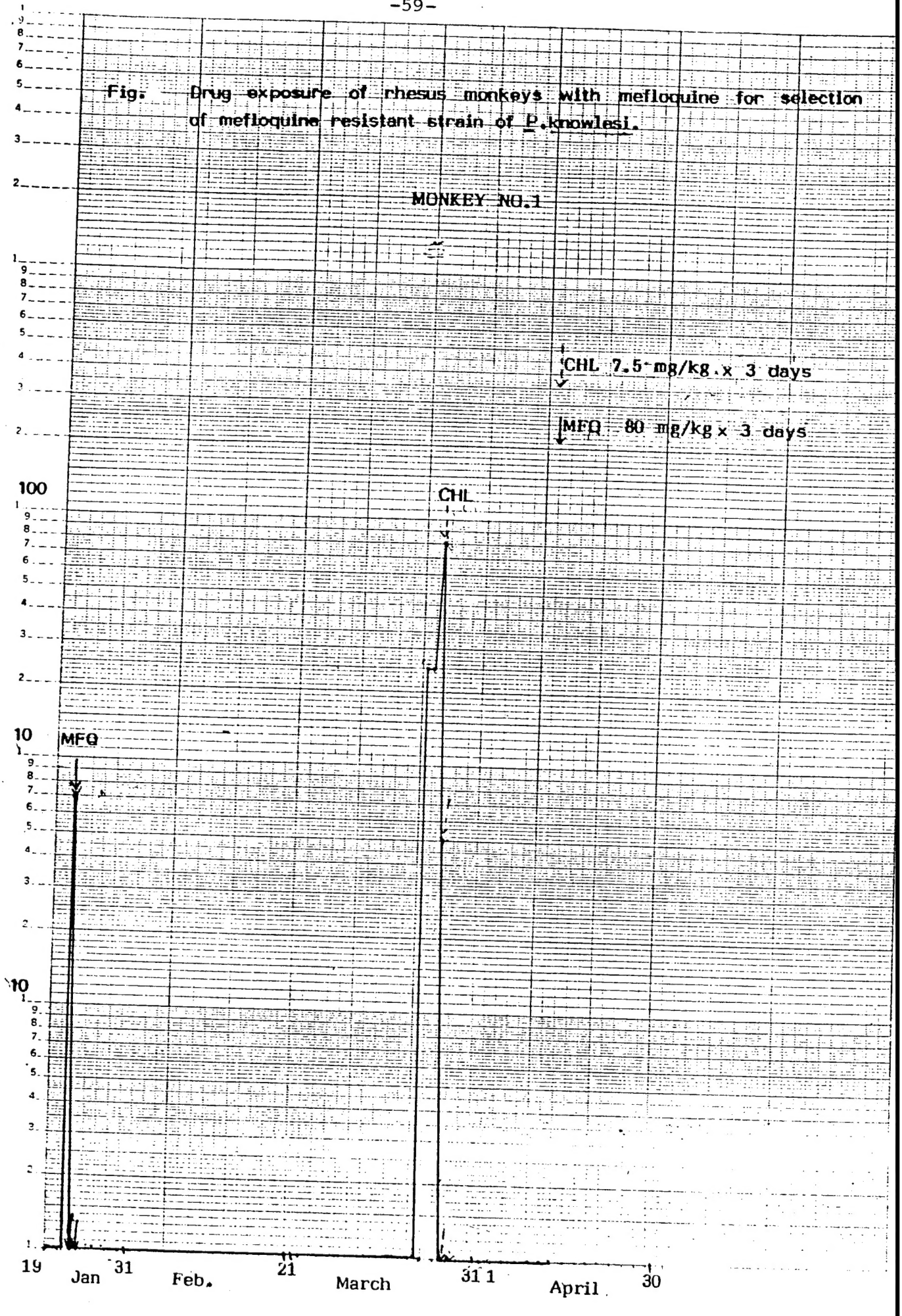


Fig. Drug exposure of rhesus monkeys with mefloquine for selection of mefloquine resistant strain of P.knowlesi.

Fig.8

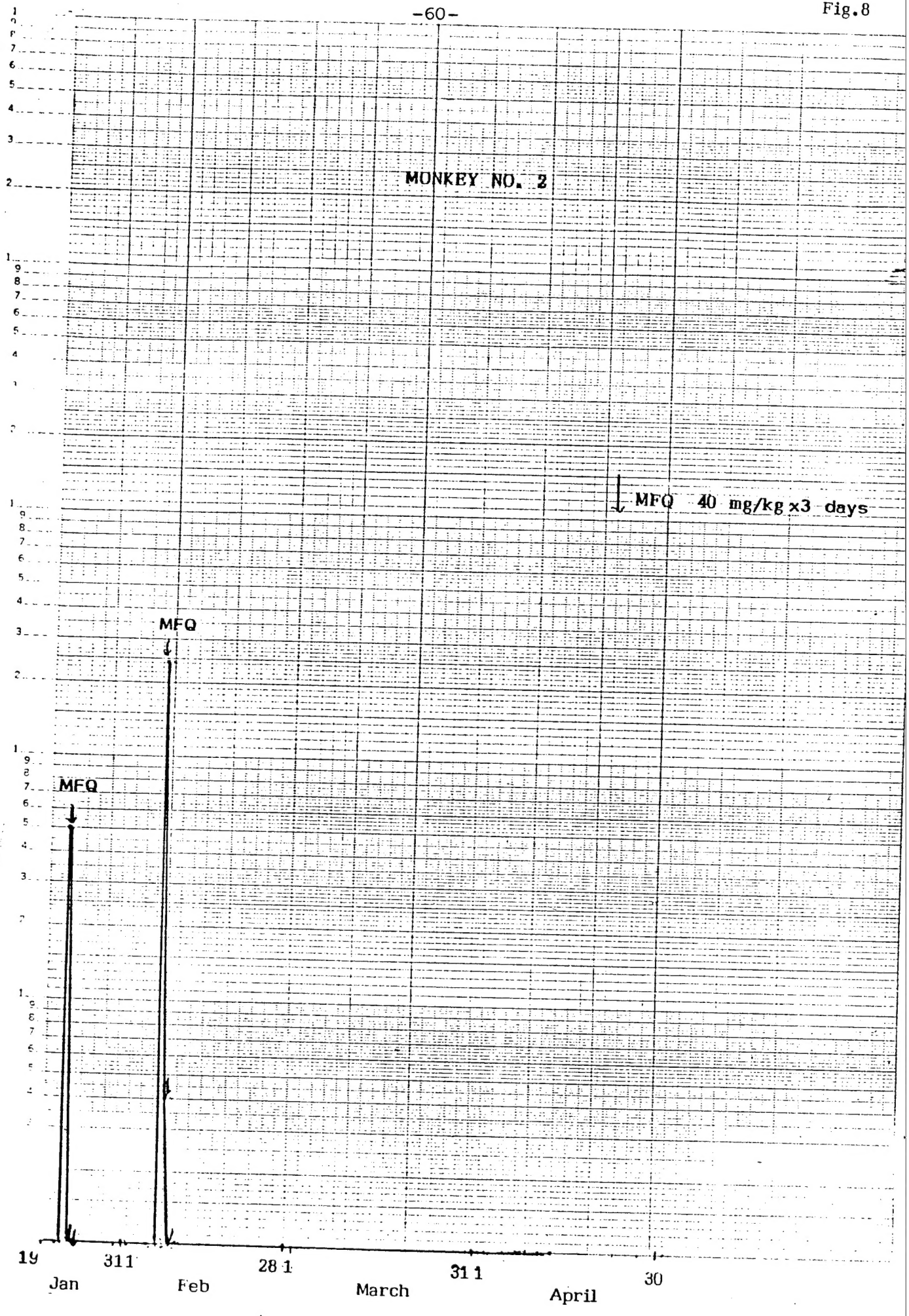

MONKEY NO. 2
MFQ 40 mg/kg x3 days
MFQ
MFQ
19
311
Jan
28 1
Feb
31 1
March
30
April

Fig.9.